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(54) Title: CALCIUM CHANNEL BLOCKING POLYPEPTIDES FROM THERAPHOSIDAE APHONOPELMA

(57) Abstract

Polypeptides isolated from the venom of the *Theraphosidae aphonopelma* spider block calcium channels in cells of various organisms and are useful in blocking said calcium channels in cells, per se, in the treatment of calcium channel-mediated diseases and conditions and in the control of invertebrate pests.

CALCIUM CHANNEL BLOCKING POLYPEPTIDES FROM THERAPHOSIDAE APHONOPELMA

Background of the Invention

This invention relates to polypeptides found in the venom of the Theraphosidae aphonopelma spider and to polypeptides having substantially the same amino acid sequence and substantially the same activity as said polypeptides. The polypeptides and the pharmaceutically acceptable salts thereof block calcium channels in cells including neuronal and muscle cells of various organisms including invertebrates and vertebrates. This invention also relates to the use of said polypeptides and their salts in blocking calcium channels in cells such as cells in the nervous and muscular system of an organism, per se, and in the treatment of calcium channel mediated diseases and conditions in a mammal. Further, this invention relates to compositions comprising said polypeptides and salts thereof.

Compounds which are calcium antagonists have a variety of utilities. Calcium antagonists can find clinical application in the treatment of such conditions as angina, hypertension, cardiomyopathies, supraventricular arrhythmias, aesophogeal achalasia, premature labor and Raynaud's disease among others. See W. G. Nayler, Calcium Antagonists, Academic Press, Harcourt Brace Jovanovich Publishers, New York, NY 1988, the teachings of which are incorporated herein by reference. Further, such compounds are useful in the study of the physiology of cells such as neuronal and muscle cells.

Summary of the Invention

This invention concerns polypeptides found in the venom of the Theraphosidae aphonopelma spider. The polypeptides of this invention and the fractions in which they are present according to this invention are as follows.

Aphonopelma peptide 6-6 has the amino acid sequence, SEQ ID NO:1.
Aphonopelma peptide 6-8 has the amino acid sequence, SEQ ID NO:2.
Aphonopelma peptide 7-6.1 has the amino acid sequence, SEQ ID NO:3.
Aphonopelma peptide 7-13.1 has the amino acid sequence, SEQ ID NO:4.
Aphonopelma peptide 7-13.2 has the amino acid sequence, SEQ ID NO:5.
Aphonopelma peptide 7-15.2 has the amino acid sequence, SEQ ID NO:6.
Aphonopelma peptide 7-17.1 has the amino acid sequence, SEQ ID NO:7.
Aphonopelma peptide 7-17.3 has the amino acid sequence, SEQ ID NO:8.
Aphonopelma peptide 7-17.4 has the amino acid sequence, SEQ ID NO:9.

The polypeptides of this invention block calcium channels in cells. Accordingly, these polypeptides are useful in blocking calcium channels in cells, per se. These polypeptides are also useful in the control of invertebrate pests and in the treatment of diseases and conditions in a mammal mediated by calcium channel function in cells.

Also within the scope of this invention are polypeptides which have substantially the same amino acid sequence and substantially the same calcium channel blocking activity as the polypeptides described above.

This invention also concerns pharmaceutical compositions comprising said polypeptides and methods of administering said polypeptides.

Detailed Description of the Invention

Venom is obtained from the Theraphosidae aphonopelma spider through the process of milking by electrical stimulation according to standard methods well known to those skilled in the art. It is preferred that the method employed is one which safeguards against contamination of the whole venom by abdominal regurgitant or hemolymph. Such methods are well known to those skilled in the art. The whole venom so obtained is stored in a frozen state at about -78°C until used for purification as described below. Purification of the constituents from the whole venom is accomplished by reverse phase high performance liquid chromatography (HPLC) on a variety of preparative and semi-preparative columns such as C-4 and C-18 Vydac® columns (Rainin Instrument Co. Inc., Mack Road, Woburn

Massachusetts 01801). Peak detection is carried out monochromatically at 220-230 nm. Further analysis of the fractions can be accomplished with, for example, polychrome UV data collected with a Waters 990 diode array detector (Millipore Corporation, Waters Chromatography Division, 34 Maple Street, Milford, Massachusetts 01757). The fractions from the columns are collected by known methods such as through the use of an ISCO/"FOXY" fraction collector and an ISCO 2159 peak detector (ISCO, 4700 Superior, Lincoln, Nebraska 68504). The fractions are collected in appropriately sized vessels such as sterile polyethylene laboratoryware. Concentration of the fractions is then accomplished by lyophilization from the eluant followed by lyophilization from water. Purity of the resulting constituent fractions then can be determined by chromatographic analysis using an analytical column with a gradient system which is more isocratic than the system used in the final purification of the fractions.

The polypeptides of the invention can be sequenced according to known methods. A general strategy for determining the primary structure includes, for example, the following steps. 1) Reduction and S-pyridylation of disulfide-bridged cysteine residues to enhance substrate susceptability to enzymatic attack. 2) Controlled cleavage of the peptide through single or multi-step enzymatic digestion. 3) Isolation and purification of peptide fragments via reverse phase high performance liquid chromatography (HPLC). 4) Characterization of peptide fragments through N-terminal sequencing and ion-spray mass spectrometry.

S-pyridylethylation of cysteine residues of the polypeptides under study can be performed, for example, in solution followed by amino acid sequencing of the polypeptides. One such procedure for S-pyridylethylation can be accomplished as described below.

About 1 to 10 μg of polypeptide is dissolved or diluted in up to 50 μl of a buffer prepared by mixing 1 part 1M TrisHCl, pH 8.5, containing 4 mM EDTA and 3 parts 8M guanidine·HCl. 2.5 μl of 10% aqueous 2-mercaptoethanol is added and the mixture is incubated at room temperature in the dark under argon for two hours. After incubation, 2 μl of 4-vinylpyridine (fresh reagent stored under argon at -20°C) is added and the mixture is incubated for another two hours at room temperature in

the dark under argon. The mixture is then desalted, preferably by chromatography on a short, reverse phase column. The recovered alkylated polypeptide is then sequenced according to known methods.

Given the benefit of the disclosure herein with respect to the peptides present in fractions 6-6, 6-8, 7-6.1, 7-13.1, 7-13.2, 7-15.2, 7-17.1, 7-17.3 and 7-17.4 of venom from Theraphosidae aphonopelma, it is now possible to obtain said peptides by methods other than through isolation/purification from whole venom. The polypeptides of this invention can be produced using recombinant DNA techniques through the cloning of a coding sequence for said polypeptides or portions thereof. For example, hybridization probes which take advantage of the now known amino acid sequence information of said polypeptides can be employed according to methods well known to those skilled in the art to clone a coding sequence for the entire polypeptide. A combination of recombinant DNA techniques and in vitro protein synthesis can also be employed to produce the polypeptides of this invention. Such in vitro protein synthesis methods include, but are not limited to, use of an ABI 430A solid phase peptide synthesizer (Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, California 94404) employing standard Merrifield chemistry or other solid phase chemistries well known to those skilled in the art.

It is well known in the art that certain amino acid substitutions can be made in polypeptides which do not affect, or do not substantially affect, the function of said polypeptides. The exact substitutions which are possible vary from polypeptide to polypeptide. Determination of permissible substitutions is accomplished according to procedures well known to those skilled in the art. Thus, all polypeptides having substantially the same amino acid sequence and substantially the same calcium channel blocking activity are within the scope of this invention.

The polypeptides of this invention block calcium channels present in a variety of cells such as cells in the nervous and muscular system of invertebrates and vertebrates.

The ability of the polypeptides of this invention to block calcium channels is demonstrated by the following procedure. Cerebellar granule cells are prepared from the cerebellum of 8 day old rats (Wilkin et al., Brain Res. **115**, 181-199, 1976). Squares (1 cm^2) of Aclar (Proplastics Inc., 5033 Industrial Ave., Wall, NJ 07719) are coated with poly-L-lysine and placed in 12-well dishes that contain 1 ml of Eagles Basal Medium. The cells are dissociated and aliquots containing 6.25 x 10^6 cells are added to each well containing the squares of Aclar. Cytosine-beta-D-arabino furanoside (final concentration 10 μM) is added 24 hours after plating. The cells are used for fura2 analysis at 6, 7 and 8 days of culture. The cells (attached to the Aclar squares) are transferred to 12 well dishes containing 1 ml of 2 μM fura2/AM (Molecular Probes Inc., Eugene, OR 97402) in HEPES buffer (containing 0.01% bovine serum albumin, 0.01% dextrose, pH 7.4, magnesium-free). The cells are incubated for 40 minutes at 37°C; the fura2/AM-containing buffer is removed and replaced with 1 ml of the same buffer without fura2/AM. To a quartz cuvette is added 2.0 ml of prewarmed (37°C) buffer. The cells on the Aclar are placed in the cuvette and the cuvette is inserted in a thermostatted (37°C) holder equipped with a magnetic stirrer and the fluorescence is measured with a fluorescence spectrophotometer (Biomedical Instrument Group, University of Pennsylvania). The fluorescence signal is allowed to stabilize for about two minutes. Then 5-20 μl of a stock solution of the compound under study in phosphate buffered saline (PBS, pH 7.4) at appropriate concentration is added to the cuvette. Calibration of the fluorescent signals and fura2/AM leakage correction are performed using the established procedures of Nemeth et al., J. Biol. Chem., **262**, 5188 (1987) at the completion of each test. The maximum fluorescence value (Fmax) is determined by addition of ionomycin (35 μM) and the minimum fluorescence value (Fmin) is determined by the subsequent addition of EGTA (12 mM) to chelate calcium. Employing the foregoing procedure, calcium channel blocking by a subject polypeptide is shown to occur by a decrease in fluorescence upon addition of the subject polypeptide. The polypeptides of the invention exhibit low IC_{50} values, under 200 nm, for blocking calcium channels using this assay. For comparison, two known commercial calcium channel antagonists, Nifedipine and Verapamil, have IC_{50} values of 33 nm and 4800 nm, respectively.

The polypeptides of this invention are useful as calcium channel blockers in cells, per se. As such, these polypeptides are also useful in the control of invertebrate pests and in the treatment of diseases and conditions mediated by calcium channels function in cells in a mammal such as angina, hypertension, cardiomyopathies, supraventricular arrhythmias, aesophogeal achalasia, premature labor and Raynaud's disease. Further, these polypeptides are useful in the study of the physiology of cells including, but not limited to, cells of the nervous, muscular and cardiovascular system.

Also within the scope of this invention are the pharmaceutically acceptable salts of the polypeptides of this invention. Such salts are formed by methods well known to those skilled in the art. For example, acid salts of the polypeptides can be prepared according to conventional methods.

When a polypeptide of this invention is to be administered to a mammal, it can be administered alone or in combination with pharmaceutically acceptable carriers or diluents in a pharmaceutical composition according to standard pharmaceutical practice. The polypeptides can be administered orally or parenterally with the parenteral route of administration being preferred for polypeptides. Parenteral administration includes intravenous, intramuscular, intraperitoneal, subcutaneous and topical administration.

For oral use of a polypeptide of this invention, the compound can be administered, for example, in the form of tablets or capsules, or as an aqueous solution or suspension. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch, and lubricating agents, such as magnesium stearate, are commonly added. For oral administration in capsule form, useful diluents are lactose and dried corn starch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, sterile solutions of the active ingredient are usually prepared, and the pH of the solutions

should be suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

When a polypeptide or salt thereof of this invention is used in a human subject, the daily dosage will normally be determined by the prescribing physician. Moreover, the dosage will vary according to the age, weight and response of the individual patient, as well as the severity of the patient's symptoms and the potency of the particular compound being administered.

When a polypeptide or salt thereof of this invention is used in control of invertebrate pests, said polypeptide is administered to said invertebrate directly or provided to the environment of said invertebrate. For example, a compound of this invention can be sprayed as a solution onto said invertebrate. The amount of compound necessary for control of said invertebrate will vary according to the invertebrate and environmental conditions and will be determined by the person applying the compound.

When a polypeptide or salt thereof of this invention is used in the physiological study of cells, said polypeptide is administered to the cells according to methods well known to those skilled in the art. For example, said polypeptide can be administered to cells in an appropriate physiological buffer. An appropriate concentration of a polypeptide of this invention for use in such studies is 200 μM. However, the concentration of said polypeptide in such studies may be greater than or much less than 200 μM. The amount of the polypeptide administered will be determined by the person skilled in the art according to well known methods.

Examples

Step I, Fractionation of Crude Aphonopelma Venom

Crude Theraphosidae aphonopelma venom (~40 μl) was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 22 x 250 mm) and was operated using a biphasic linear gradient program from 95% A and 5% B to 80% A and 20% B over 30 minutes, then from 80% A and 20% B to 30% A and 70% B over 25 minutes (A = 0.1% trifluoroacetic acid (TFA), B = acetonitrile) with detection at 220 nm and a flow rate of 15 ml/minute. Fractions were collected as set forth below.

TABLE 1

Fraction 6	35.9 to 37.3 minutes.
Fraction 7	37.3 to 39.5 minutes.
Fraction 8	39.5 to 40.1 minutes.
Fraction 9	40.1 to 40.9 minutes.
Fraction 10	40.9 to 41.8 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization. Further fractionations were performed as described below.

Step II. Subfractionation of Step I Fractions

A) Subfractionation of Fraction 6

The Fraction 6 material from Step 1, above, derived from ~60 μl of crude venom, was applied to a reversed phase HPLC column (Baker WP C-18, 4.6 x 250 mm) and was operated using a linear gradient program from 85% A and 15% B to 65% A and 35% B over 45 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 1.0 ml/minute. Fractions were collected as set forth below.

TABLE 2

Fraction 6-6	24.0 to 24.8 minutes.
Fraction 6-7	25.0 to 25.4 minutes.
Fraction 6-8	25.6 to 26.5 minutes.
Fraction 6-9	27.0 to 28.7 minutes.
Fraction 6-13	33.5 to 34.3 minutes.
Fraction 6-14	34.4 to 35.4 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization.

B) Subfractionation of Fraction 7

The Fraction 7 material from Step 1, above, derived from ~60 μl of crude venom, was applied to a strong cation exchange column (Sulfoethyl aspartamide (The Nest Group, 45 Valley Rd., Southborough, MA 01772), 5 μ, 4.6 x 200 mm) and was operated using a linear gradient program from 100% A and 0% B to 0% A and 100% B over 45 minutes (A = 5 mM H_3PO_4/20% acetonitrile, B = 5 mM H_3PO_4, 1.0

M NaCl/20% acetonitrile) with detection at 230 nm and a flow rate of 1.0 ml/minute. Fractions were collected as set forth below.

TABLE 3

Fraction 7-6	21.9 to 22.3 minutes.
Fraction 7-9	24.8 to 25.5 minutes.
Fraction 7-11	26.1 to 26.7 minutes.
Fraction 7-12	27.1 to 27.6 minutes.
Fraction 7-13	27.6 to 28.9 minutes.
Fraction 7-14	29.7 to 30.8 minutes.
Fraction 7-15	31.0 to 32.3 minutes.
Fraction 7-16	32.5 to 33.1 minutes.
Fraction 7-17	34.2 to 36.0 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization.

C) Subfractionation of Fraction 8

The Fraction 8 material from Step 1, above, derived from ~100 µl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using a linear gradient program from 80% A and 20% B to 71% A and 29% B over 35 minutes, then 71% A and 29% B for 10 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 6.0 ml/minute. Fractions were collected as set forth below.

TABLE 4

Fraction 8-5	19.2 to 21.2 minutes.
Fraction 8-6	21.3 to 22.5 minutes.
Fraction 8-7	25.9 to 26.5 minutes.
Fraction 8-8	27.4 to 28.3 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization.

Step III. Subfractionation of Step II fractions

A) Subfractionation of Fraction 7-6

The Fraction 7-6 material from Step II-B, above, derived from ~70 µl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using a linear gradient program of 90% A and 10% B for

10 minutes, then from 90% A and 10% B to 60% A and 40% B over 40 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fractions were collected as set forth below.

TABLE 5

Fraction 7-6.1	32.2 to 34.3 minutes.
Fraction 7-6.2	35.5 to 36.2 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization.

B) Subfractionation of Fraction 7-9

The Fraction 7-9 material from Step II-B, above, derived from ~300 µl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using a linear gradient program of 90% A and 10% B for 10 minutes, then from 90% A and 10% B to 65% A and 35% B over 40 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fraction 7-9.1 was collected and pooled from multiple runs with an elution time from 33 to 35 minutes.

C) Subfractionation of Fraction 7-11

The Fraction 7-11 material from Step II-B, above, derived from ~600 µl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using a linear gradient program of 90% A and 10% B for 10 minutes, then from 90% A and 10% B to 65% A and 35% B over 60 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fraction 7-11.1 was collected and pooled from multiple runs with an elution time from 34.2 to 35.5 minutes.

D) Subfractionation of Fraction 7-12

The Fraction 7-12 material from Step II-B, above, derived from ~600 µl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using 90% A and 10% B for 10 minutes, then a linear gradient program from 90% A and 10% B to 65% A and 35% B over 60

minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fractions were collected as set forth below.

TABLE 6

Fraction 7-12.1	28.0 to 29.1 minutes.
Fraction 7-12.2	38.4 to 39.6 minutes.

E) Subfractionation of Fraction 7-13

The Fraction 7-13 material from Step II-B, above, derived from ~200 µl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using 90% A and 10% B for 10 minutes, then a linear gradient program from 90% A and 10% B to 60% A and 40% B over 40 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fractions were collected as set forth below.

TABLE 7

Fraction 7-13.1	33.0 to 34.5 minutes.
Fraction 7-13.2	35.1 to 36.5 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization.

F) Subfractionation of Fraction 7-15

The Fraction 7-15 material from Step II-B, above, derived from ~200 µl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using 90% A and 10% B for 10 minutes, then a linear gradient program from 90% A and 10% B to 60% A and 40% B over 40 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fractions were collected as set forth below.

TABLE 8

Fraction 7-15.1	33.1 to 33.6 minutes.
Fraction 7-15.2	34.5 to 36.2 minutes.
Fraction 7-15.3	37.7 to 39.1 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization.

G) Subfractionation of Fraction 7-16

The Fraction 7-16 material from Step II-B, above, derived from ~600 μl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using 90% A and 10% B for 10 minutes, then a linear gradient program from 90% A and 10% B to 65% A and 35% B over 60 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fraction 7-16.1 was collected and pooled from multiple runs with an elution time from 31.8 to 33.0 minutes.

H) Subfractionation of Fraction 7-17

The Fraction 7-17 material from Step II-B, above, derived from ~25 μl of crude venom, was applied to a reversed phase HPLC column (Vydac®, C-18, 300 Å, 10 x 250 mm) and was operated using 90% A and 10% B for 10 minutes, then a linear gradient program from 90% A and 10% B to 60% A and 40% B over 40 minutes (A = 0.1% TFA, B = acetonitrile) with detection at 220 nm and a flow rate of 3.5 ml/minute. Fractions were collected as set forth below.

TABLE 9

Fraction 7-17.1	15.6 to 18.4 minutes.
Fraction 7-17.2	18.5 to 19.4 minutes.
Fraction 7-17.3	27.1 to 28.6 minutes.
Fraction 7-17.4	28.6 to 30.1 minutes.
Fraction 7-17.5	30.1 to 31.9 minutes.
Fraction 7-17.6	31.9 to 33.5 minutes.

Each of the above fractions, from pooled runs, was concentrated by lyophilization.

Example 1 Aphonopelma peptide 6-6

The structure of peptide 6-6, prepared in Step II-A, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing

was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 6-6 as shown below.

SEQ ID NO:1, 39 residues, 6 cysteines, 3 disulfide bonds.
Calculated mass = 4382.3.
Observed mass = 4382.16 ± 0.54 (ion spray m.s.).

Example 2 Aphonopelma peptide 6-8

The structure of peptide 6-8, prepared in Step II-A, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 6-8 as shown below.

SEQ ID NO:2, 39 residues, 6 cysteines, 3 disulfide bonds.
Calculated mass = 4369.2.
Observed mass = 4368.26 ± 0.27 (ion spray m.s.).

Example 3 Aphonopelma peptide 7-6.1

The structure of peptide 7-6.1, prepared in Step III-A, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 7-6.1 as shown below.

SEQ ID NO:3, 33 residues, 6 cysteines, 3 disulfide bonds.

Calculated mass = 3786.2.

Observed mass = 3784.54 (ion spray m.s.).

Example 4 Aphonopelma peptide 7-13.1

The structure of peptide 7-13.1, prepared in Step III-E, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 7-13.1 as shown below.

SEQ ID NO:4, 34 residues, 6 cysteines, 3 disulfide bonds.

Calculated mass = 3814.32.

Observed mass = 3813.67 ± 0.27 (ion spray m.s.).

Example 5 Aphonopelma peptide 7-13.2

The structure of peptide 7-13.2, prepared in Step III-E, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 7-13.2 as shown below.

SEQ ID NO:5, 42 residues, 6 cysteines, 3 disulfide bonds.

Calculated mass = 4844.45.

Observed mass = 4844.66 (ion spray m.s.).

Example 6 Aphonopelma peptide 7-15.2

The structure of peptide 7-15.2, prepared in Step III-F, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 7-15.2 as shown below.

SEQ ID NO:6, 39 residues, 6 cysteines, 3 disulfide bonds.
Calculated mass = 4342.19.
Observed mass = 4341.84 ± 0.33 (ion spray m.s.).

Example 7 Aphonopelma peptide 7-17.1

The structure of peptide 7-17.1, prepared in Step III-H, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 7-17.1 as shown below.

SEQ ID NO:7, 39 residues, 6 cysteines, 3 disulfide bonds.
Calculated mass = 4383.28.
Observed mass = 4382.33 ± 0.52 (ion spray m.s.).

Example 8 Aphonopelma peptide 7-17.3

The structure of peptide 7-17.3, prepared in Step III-H, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal

sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 7-17.3 as shown below.

SEQ ID NO:8.
Observed mass = 4368.23 ± 0.47 (ion spray m.s.).

Example 9 Aphonopelma peptide 7-17.4

The structure of peptide 7-17.4, prepared in Step III-H, above, was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

The data taken together affirm the structure of peptide 7-17.4 as shown below.

SEQ ID NO:9, 39 residues, 6 cysteines, 3 disulfide bonds.
Calculated mass = 4383.23.
Observed mass = 4382.19 ± 0.38 (ion spray m.s.).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Pfizer Inc
(B) STREET: 235 East 42nd Street
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 10017
(G) TELEPHONE: (203) 441-4905
(H) TELEFAX: (203) 441-5221

(A) NAME: NPS Pharmaceuticals, Inc.
(B) STREET: 420 Chipeta Way
(C) CITY: Salt Lake City
(D) STATE: Utah
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 84108
(G) TELEPHONE: (801) 583-4939
(H) TELEFAX: (801) 583-4961

(ii) TITLE OF INVENTION: CALCIUM CHANNEL BLOCKING POLYPEPTIDES FROM THERAPHOSIDAE APHONOPELMA

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/973,323
(B) FILING DATE: 03-NOVEMBER-1992

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Leu Phe Glu Cys Val Leu Ser Cys Asp Ile Lys Lys Asn Gly Lys Pro
1               5                   10                  15

Cys Lys Pro Lys Gly Glu Lys Lys Cys Ser Gly Gly Trp Arg Cys Lys
            20                  25                  30

Ile Asn Phe Cys Leu Lys Val
        35
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Phe Glu Cys Ala Leu Ser Cys Asp Ile Lys Lys Asn Gly Lys Pro
1 5 10 15

Cys Lys Pro Lys Gly Glu Lys Lys Cys Ser Gly Gly Trp Arg Cys Lys
20 25 30

Ile Asn Phe Cys Leu Lys Ile
35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys Ala Glu Phe Gln Ser Lys Cys Lys Lys Asp Ser Glu Cys Cys Gly
1 5 10 15

Thr Leu Glu Cys Ser Pro Thr Trp Lys Trp Cys Val Tyr Pro Ser Pro
20 25 30

Phe

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ser Cys Gly His Val Gly Thr Pro Cys Glu Lys Asn Trp Asp Cys Cys
1               5                   10                  15

Lys Gly Lys Val Cys Ser Pro Arg Trp Lys Leu Cys Ala Tyr Glu Ser
            20                  25                  30

Pro Phe
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Cys Leu Gly Glu Asn Val Pro Cys Asp Lys Asp Arg Pro Asn Cys Cys
1               5                   10                  15

Ser Lys Tyr Glu Cys Leu Glu Pro Thr Gly Tyr Gly Arg Cys Tyr Ala
            20                  25                  30

Ser Tyr Tyr Ser Tyr Lys Lys Lys Thr Leu
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Leu Ile Glu Cys Ala Phe Ser Cys Asp Ile Thr Lys Asn Gly Lys Pro
1               5                   10                  15

Cys Lys Pro Lys Gly Glu Lys Lys Cys Ser Gly Gly Trp Arg Cys Lys
            20                  25                  30

Ile Asn Phe Cys Leu Lys Ile
        35
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Leu Phe Glu Cys Val Leu Ser Cys Asp Ile Lys Lys Asn Gly Lys Pro
1               5                   10                  15

Cys Lys Pro Lys Gly Glu Lys Lys Cys Ser Gly Gly Trp Arg Cys Lys
            20                  25                  30

Ile Asn Phe Cys Leu Lys Val
        35
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma

(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Phe Glu Cys Ala Leu Ser Cys Asp Ile Lys Lys Asn Gly Lys Pro
1 5 10 15

Cys Lys Pro Xaa Gly Glu Lys Lys Cys Ser Gly Gly Xaa Arg Xaa Xaa
20 25 30

Ile Asn Phe
35

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theraphosidae aphonopelma
(F) TISSUE TYPE: venom

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Phe Glu Cys Val Leu Ser Cys Asp Ile Lys Lys Asn Gly Lys Pro
1 5 10 15

Cys Lys Pro Lys Gly Glu Lys Lys Cys Ser Gly Gly Trp Arg Cys Lys
20 25 30

Ile Asn Phe Cys Leu Lys Val
35

CLAIMS

1. A substantially pure polypeptide comprising the amino acid sequence, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 or a polypeptide having substantially the same amino acid sequence and substantially the same calcium channel blocking activity as said polypeptide, or a pharmaceutically acceptable salt thereof.

2. A substantially pure polypeptide according to Claim 1 having the amino acid sequence, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 or a pharmaceutically acceptable salt thereof.

3. A method of blocking calcium channels in a cell comprising administering to said cell a calcium channel blocking amount of a polypeptide according to Claim 1.

4. A method according to Claim 3 wherein said cell is in the nervous system of a mammal.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/09069

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K7/10 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOLOGICAL CHEMISTRY HOPPE-SEYLER vol. 370, no. 5 , May 1989 , BERLIN, DE pages 484 - 498 A. SAVEL-NIEMANN 'TARANTULA (EURYPELMA CALIFORNICUM) VENOM, A MULTICOMPONENT SYSTEM.' see page 495; figure 8	1-4
A	CHEMICAL ABSTRACTS, vol. 68, no. 23, 3 June 1968, Columbus, Ohio, US; abstract no. 101945w, H.L. STAHNKE ET AL. 'APHONOPELMA TARANTULA VENOM.' page 9838 ; see abstract & ANIM. TOXINS, COLLECT. PAP. INT. SYMP., 1 ST, ATLANTIC CITY 1966 pages 35 - 39	1-4

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 1994	17 -02- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 93/09069

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : although claims 3 and 4 as far as relating to an in vivo method are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark 2 : Attention is drawn to the fact that seq ID n 1 is identical to seq ID n 7 and 9.